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Impact of Environmental Factors on Bacteriocin Promoter Activity in Gut-Derived *Lactobacillus salivarius*

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Bacteriocin production is regarded as a desirable probiotic trait that aids in colonization and persistence in the gastrointestinal tract (GIT). Strains of *Lactobacillus salivarius*, a species associated with the GIT, are regarded as promising probiotic candidates and have a number of associated bacteriocins documented to date. These include multiple class IIb bacteriocins (salivaricin T, salivaricin P, and ABP-118) and the class IId bacteriocin bactofencin A, which show activity against medically important pathogens. However, the production of a bacteriocin in laboratory media does not ensure production under stressful environmental conditions, such as those encountered within the GIT. To allow this issue to be addressed, the promoter regions located upstream of the structural genes encoding the *L. salivarius* bacteriocins mentioned above were fused to a number of reporter proteins (green fluorescent protein [GFP], red fluorescent protein [RFP], and luciferase [Lux]). Of these, only transcriptional fusions to GFP generated signals of sufficient strength to enable the study of promoter activity in *L. salivarius*. While analysis of the class IIb bacteriocin promoter regions indicated relatively weak GFP expression, assessment of the promoter of the anti-staphylococcal bacteriocin bactofencin A revealed a strong promoter that is most active in the absence of the antimicrobial peptide and is positively induced in the presence of mild environmental stresses, including simulated gastric fluid. Taken together, these data provide information on factors that influence bacteriocin production, which will assist in the development of strategies to optimize *in vivo* and *in vitro* production of these antimicrobials.

Bacteriocins are a heterogeneous family of small, ribosomally synthesized peptides with antimicrobial activity produced by many bacterial species (1–3). These antimicrobials can have a broad or narrow spectrum of activity and have considerable potential as agents in food preservation and biomedical applications. Bacteriocin production is considered an important trait of gut-derived bacteria, influencing microbial populations within the intestinal tract (for a review, see reference 4). *Lactobacillus salivarius* is a species associated with the gastrointestinal tract (GIT), with many probiotic traits. *L. salivarius* strains are frequently producers of unmodified class IIa, IIb, and IId bacteriocins (5–9). Class IIa bacteriocins are generally classified as pediocin-like peptides; class IIb comprises the two-component unmodified peptides; and class IId bacteriocins are often categorized on the basis of their dissimilarity to other class II peptides (10).

L. salivarius NCIMB 40829 (LSUCC118) is an extensively studied strain that produces the class IIb two-peptide bacteriocin ABP-118 (11). The *in vivo* functionality of the ABP-118 bacteriocin and its effectiveness in eliminating a GIT pathogen in a mouse model have been demonstrated previously (12). Closely related variants of ABP-118, such as salivaricin P, and indeed other two-peptide bacteriocins, such as salivaricin T, have also been isolated from several intestinal *L. salivarius* strains (7, 13). The production of these bacteriocins and immunity to them rely on the coordinated expression of at least 12 genes (8, 11, 13). Recent work has led to the identification of a novel class IId bacteriocin, bactofencin A, produced by *L. salivarius* isolates from porcine intestines (9). This bacteriocin gene cluster consists of just 4 genes, including the structural peptide BfnA, which shows little identity to previously isolated bacteriocins but shares some similarity with eukaryotic cationic peptides. This bacteriocin demonstrated antimicrobial activity against medically important pathogens, including *Staphylococcus aureus* (9). The level of *in vivo* production of bactofencin A, and indeed those of many other bacteriocins, and the ways in which their production is influenced by stressful environmental conditions are as yet unknown, even though it has been documented that bacteriocin production can be sensitive to environmental changes and to parameters including temperature, pH, and the growth medium (14–16). Research on these issues is critical for the successful use of bacteriocin-producing strains for food and/or medical applications.

With respect to what is known already, it is clear that the regulation of bacteriocin production can be complex and in some cases involves a quorum-sensing, cell density-dependent mechanism that relies on a pheromone-like peptide and a cognate two-component regulatory system (17). The inducing peptides (IP), thought to be produced at a low basal level in early growth, can reach a critical threshold concentration either due to an accumulative process or by increased production elicited by an environmental stimulus. Once the required IP level is reached, the signal is processed by the two-component system, which binds to the promoters of the bacterial structural genes to allow bacteriocin pro-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Source or reference(s)
Strains		
E. coli DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*; host for pNZ44 fluorescent derivatives	Life Technologies
L. salivarius		
UCC118	Human ileocecal isolate; ABP-118 bacteriocin producer	11
DPC 6488	Human isolate; salivaricin TL producer	8
DPC 6502	Porcine isolate; bactofencin A producer	9
DPC 6189	Porcine isolate; salivaricin P and bactofencin A producer	8, 13
L. delbrueckii subsp. *bulgaricus* LMG 6901	Bacteriocin-sensitive indicator	13
Plasmids		
pNZ44	Cm^r; lactococcal expression vector	41
pEVS*gfp*+	Source of *gfp* gene	28
pDsRed express vector	Amp^r; *lacZ*-DsRed-Express fusion protein	Clontech
pPl2Lux	Derivative of the listerial integration vector pPL2; harbors the synthetic *luxABCDE* genes	30

[a] Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

duction (17). These regulatory mechanisms and the promoter elements involved have been studied in depth for the plantaricin (18, 19) and sakacin (20, 21) gene clusters. In some cases, most notably in lantibiotic (class I) gene clusters, the bacteriocin itself can function as the inducing peptide (22).

Of the salivaricins that are the focus of our studies, the 3 class IIb nonlantibiotic bacteriocins (ABP-118, salivaricin P, and salivaricin T) are predicted to have an IP-associated regulatory mechanism similar to that described above (11), whereas the gene cluster associated with the class IId bacteriocin bactofencin A does not contain an obvious regulatory mechanism (9). With a view to identifying the environmental factors that influence the production of these bacteriocins, the putative bacteriocin promoter regions were fused to a reporter gene in order to detect promoter activity under various environmental conditions. More specifically, a stable expression system using green fluorescent protein (GFP) was established, and the promoter-*gfp* fusions were monitored in *L. salivarius* bacteriocin-producing backgrounds. Promoter activity was assessed under a number of environmental conditions, some of which simulate the stressful environment of the GIT. The knowledge thus obtained will allow the development of strategies to optimize the production of these bacteriocins *in vivo* and *in vitro* and will provide valuable fundamental insights to facilitate similar experiments with other bacteriocin-producing microbes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Escherichia coli* and *L. salivarius* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium at 37°C. *L. salivarius* strains were cultured under anaerobic conditions in MRS broth (Difco Laboratories, Detroit, MI) at 37°C for 24 to 48 h, except for fluorescence expression analysis, for which cells were grown statically at 37°C in order to achieve microaerobic conditions. Ampicillin was added at 50 μg/ml, and chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was added at 10 μg/ml and 5 μg/ml for *E. coli* and *L. salivarius* strains, respectively.

***In silico* analysis of putative promoter regions.** The bacteriocin gene clusters of salivaricin P (13), salivaricin T (8), ABP-118 (11), and bactofencin A (9) were analyzed using Artemis software (23). Regions upstream of the structural peptides and prepeptides were investigated for putative promoter regions by using BPROM (24) and Virtual Footprint (version 3.0) (25) and by manual annotation of the operons. Direct repeats were searched for by using the Tandem Repeats Finder software (26).

DNA manipulations. The primers used for PCR were purchased from Sigma-Aldrich (St. Louis, MO, USA) and are listed in Table S1 in the supplemental material. Platinum *Taq* DNA polymerase (Life Technologies, Carlsbad, CA, USA) or MyTaq Red Mix (Bioline, London, United Kingdom) was used for PCR amplifications. Restriction enzymes, Klenow DNA polymerase I, and T4 DNA ligase were all purchased from Roche (Manheim, Germany) and were used as outlined in the manufacturer's instructions. PCR purification, gel extraction, and plasmid preparation kits were obtained from Qiagen (Venlo, Netherlands) and were used as specified by the manufacturers. The genomic DNA of *L. salivarius* strains was extracted as described previously (27).

Construction of expression plasmids. The pNZ44 plasmids expressing reporter protein GFP or DsRed (red fluorescent protein [RFP]), or the Lux (luciferase) system, were created as follows. The *gfp*$^+$ gene including a ribosome binding site (RBS) was amplified from plasmid pEVS*gfp*+ (28) by using primers *gfp*+For and *gfp*+Rev (see Table S1 in the supplemental material) containing the PstI and XbaI restriction sites. The cloning vector pNZ44 and the *gfp* insert were digested with the restriction enzymes PstI and XbaI and were ligated together, and the resulting pNZ44.*gfp*+ plasmid was transformed first into chemically competent *E. coli* DH10B cells (Life Technologies, Carlsbad, CA, USA) and subsequently into *L. salivarius* cells. The DsRed reporter gene (*rfp*) was amplified from the pDsRed-Express vector (Clontech, Mountain View, CA, USA) by using primers DsRed+For and DsRed+Rev (see Table S1) to create the pNZ44.*rfp*+ plasmid. These primers incorporated an RBS site and altered start codons to reduce the GC content at the beginning of the gene so as to increase the likelihood of translation initiation, as recommended previously (29). The luciferase genes were amplified from the pP2lux plasmid (30) with the *lux*+For and *lux*+Rev primers and were also cloned into the PstI and XbaI sites of pNZ44 to create the pNZ44.*lux*+ plasmid (Table 2). In certain *gfp*$^+$ plasmids, the p44 promoter was replaced with a constitutive lactobacillus promoter (p*cysK*). Subsequently, putative promoter regions for the bacteriocin operons encoding bactofencin A, salivaricin P, salivaricin T, and ABP-118 were amplified from *L. salivarius* genomic DNA and were cloned, using the BglII and PstI restriction sites, to create transcriptional fusions to the *gfp*$^+$ gene. The constructs created are described in Table 2; the primers used to create the amplicons for cloning are described in Table S1 in the supplemental material. To generate the 151-bp putative promoter region representing

TABLE 2 Fluorescence-expressing plasmids constructed in this study

Plasmid	Relevant properties
pNZ44.*gfp*	pNZ44 plasmid with the *gfp* gene fused to the p44 promoter
pNZ.gfp−	Promoterless pNZ44-derived plasmid containing the intact *gfp* gene
pNZ44.*rfp*	pNZ44 plasmid with the *rfp* gene fused to the p44 promoter
pNZ44.*lux*	pNZ44 plasmid containing the *luxABCDE* genes fused to the p44 promoter
*cysK*prom.*gfp*	pNZ44 plasmid containing the *gfp* gene; the p44 promoter is replaced with the promoter of the *cysK* gene of *L. salivarius* UCC118
*bfnA*prom.*gfp*	pNZ44 plasmid containing the *gfp* gene; the p44 promoter is replaced with the putative promoter of the bactofencin A structural gene of *L. salivarius* DPC6502
*abp-118*prom.*gfp*	pNZ44 plasmid containing the *gfp* gene; the p44 promoter is replaced with the putative promoter region upstream of the ABP-118 prepeptide of *L. salivarius* UCC118
*sal*prom1.*gfp*	pNZ44 plasmid containing the *gfp* gene; the p44 promoter is replaced with an 847-bp region upstream of the salivaricin P and T structural genes *of L. salivarius* DPC6189 and DPC6488, respectively (oligonucleotides used include *sal*prom1For and *sal*prom2Rev, in which the cloned insert begins 847 bp upstream of the start codon of the structural gene [*salT*α of DPC6488 and *sln1* of DPC6005])
*sal*prom2.*gfp*	pNZ44 plasmid containing the *gfp* gene; the p44 promoter is replaced with a 391-bp region upstream of the salivaricin P and T structural genes of *L. salivarius* DPC6189 and DPC6488, respectively (oligonucleotides used include *sal*prom2For and *sal*prom2Rev, in which the cloned insert begins 391 bp upstream of the start codon of the structural gene [*salT*α of DPC6488 and *sln1* of DPC6005])
*sal*prom3.*gfp*	pNZ44 plasmid containing the *gfp* gene; the p44 promoter is replaced with the synthesized 151-bp region[a] upstream of the salivaricin P and T prepeptides of *L. salivarius* DPC6189 and DPC6488, respectively

[a] For the sequence of the 151-bp region, see Fig. S1 in the supplemental material.

salivaricin promoter region 3 (*sal*prom3), the sequence was synthesized using the gene synthesis service of GeneWiz/Sigma-Aldrich (St. Louis, MO, USA), with BglII and PstI restriction sites included to facilitate cloning into the pNZ44.*gfp* plasmid (see Fig. S1 in the supplemental material). Plasmids were transformed into chemically competent *E. coli* DH10B cells (Life Technologies, Carlsbad, CA, USA). Following verification of the integrity of the plasmid constructs, they were transformed into electrocompetent *L. salivarius* bacteriocin-producing backgrounds (*L. salivarius* DPC6502 [producing bactofencin A], *L. salivarius* DPC6488 [producing the class IIb bacteriocins salivaricin T and salivaricin L {8}], *L. salivarius* DPC6189 [producing salivaricin P and bactofencin A], and *L. salivarius* NCIMB 40829 [LSUCC118] [producing ABP-118]) to create a bank of *gfp*$^+$ strains (see Table S2 in the supplemental material). *Lactobacillus salivarius* cells were made competent and electroporated as described previously (31). For the creation of a promoterless plasmid containing the *gfp* gene to serve as a negative control, the p44 promoter was removed from the pNZ44.*gfp* plasmid by using the BglII and PstI restriction sites. The plasmid ends were treated with Klenow DNA polymerase and were subsequently ligated with T4 DNA ligase. The integrity of the constructs was confirmed by sequencing (Beckman Coulter Genomics, Takeley, United Kingdom).

Expression of reporter genes in *L. salivarius*. To detect fluorescence in *L. salivarius* strains containing promoter-*gfp*$^+$ fusions, cells were grown overnight in broth at 37°C to stationary phase. Cells were harvested and were washed with phosphate-buffered saline (PBS), and subsequently the cell suspensions were analyzed using an epifluorescence microscope (Olympus BX-51) equipped with a fluorescein isothiocyanate filter under an Olympus UPlanFl 100× oil immersion objective lens. Images were captured with a DP50 camera (Olympus Co., Toyko, Japan) and were analyzed with Olympus analySIS software. To detect fluorescence from *L. salivarius* strains containing promoter-*rfp*$^+$ fusions, cells were prepared as described above and were analyzed using an Olympus microscope equipped with a tetramethylrhodamine isothiocyanate filter for red emission.

To detect bioluminescence, overnight cultures were inoculated at 1 to 2.5% into fresh MRS broth for *L. salivarius* cells and into LB broth for *E. coli* cells, transferred to 96-well plates, incubated, and monitored on a Xenogen IVIS 100 system (Xenogen, Alameda, CA, USA) at 37°C. The levels of bioluminescence were determined in continuous-imaging mode at high resolution with 5-min exposure times.

Growth and fluorescence assays of *gfp*$^+$ strains. Broth-based assays were performed by inoculating fully grown *L. salivarius* strains containing promoter-*gfp*$^+$ gene fusions at 2% (wt/vol) into a 0.2-ml volume of medium (MRS broth) and growing the strains statically at 37°C. The optical density at 600 nm was monitored to determine bacterial growth or to detect GFP fluorescence (excitation at 485 nm; emission at 520 nm) in a Synergy 2 spectrophotometer (BioTek, Winooski, VT, USA) over a 20-h period. Data were recorded and plotted in relative fluorescence units (RFU).

Challenge assays with environmental stimuli and stresses. Stationary-phase cells of *L. salivarius gfp*$^+$ strains were harvested by centrifugation, and cell pellets were washed with PBS and were resuspended in MRS broth. The cells were then inoculated at 2% (wt/vol) into either 1 ml MRS broth at pH 6.5, pH 6, or pH 5.5 (adjusted with 1 M HCl), MRS broth containing a 0.25%, 0.5%, or 1% (wt/vol) concentration of NaCl, or MRS broth containing 0.1%, 0.2%, or 0.3% porcine bile (Sigma-Aldrich, St. Louis, MO, USA). Aliquots (0.2 ml) were dispensed into 96-well plates and were monitored as described above.

To assess GFP expression from cells in response to the presence of target microbes, *gfp*$^+$ strains were inoculated at 1×10^9 CFU/ml into MRS broth with a bacteriocin-sensitive strain (*Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901) at 1×10^7 CFU/ml. GFP expression was monitored in 96-well plates as described above. To assess promoter activity in the presence of bacteriocin-inducing peptides (IP), the recognized IP for salivaricin P (MKFEVLTEKKLQVIVGGKQEGGTKTYDKVCRFKFLGICK) and the IP that is specific to both salivaricin T and ABP-118 (MKFEVLT EKKLQKIAGGATKKGGFKRWQCIFTFFGVCK) were synthesized using microwave-assisted solid-phase peptide synthesis (MW-SPPS), performed on a Liberty Blue microwave peptide synthesizer (CEM). Both peptides were synthesized on a 9-fluorenylmethoxy carbonyl (Fmoc)-L-Lys(Boc) HMPB-ChemMatrix resin (PCAS BioMatrix, Inc., Saint-Jean-sur-Richelieu, Quebec, Canada), purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C_8 (particle size, 10 μm; pore size, 300 Å) column (Vydac, CA, USA), and eluted using a 20- to 40% acetonitrile–0.1% trifluoroacetic acid (TFA) gradient over 40 min. The flow rate was 2.5 ml/min, and the eluent was monitored at 214 nm. Fractions containing the desired molecular mass were identified using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Shimadzu Biotech, Manchester, United Kingdom), pooled, and lyophilized on a Genevac HT-4X (Genevac Ltd.,

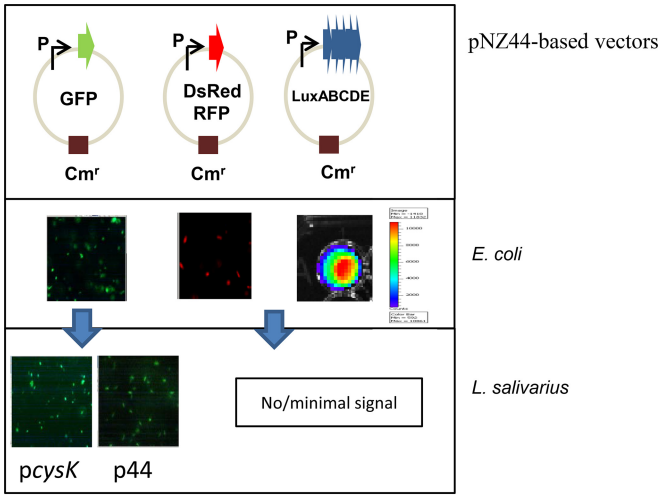

FIG 1 Schematic representation of the pNZ-based fluorescent plasmids constructed for the expression of *gfp*, *rfp*, and *lux* genes in *E. coli* and *L. salivarius* cells.

Ipswich, United Kingdom) lyophilizer. Peptides were added to harvested cells in MRS broth at 10^{-4} to 10^{-6} M, and fluorescence was monitored for 20 h as described above.

Challenge assays with simulated gastric fluid. To assess GFP expression from cells exposed to simulated gastric fluid overnight, cells were washed in PBS and were subsequently resuspended in gastric fluid for 30 s, 1 min, or 5 min. The cells were then harvested and were inoculated into MRS broth, and fluorescence was detected as described above. Simulated gastric fluid was made as described previously (32) and consisted of NaCl (2.05 g/liter), KH_2PO_4 (0.60 g/liter), $CaCl_2$ (0.11 g/liter), and KCl (0.37 g/liter), adjusted to pH 2.0 using 1 M HCl, and autoclaved at 121°C for 15 min. Porcine bile (0.05 g/liter), lysozyme (0.1 g/liter), and pepsin (0.0133 g/liter) were added as stock solutions prior to use. Components were obtained from Sigma-Aldrich (St. Louis, MO, USA).

RESULTS

Selection of a reporter system for *L. salivarius*. The GFP, RFP, and Lux reporter systems were investigated for their suitability as tools with which to study promoter activity in *L. salivarius*. Due to the potential usefulness of the Lux system for facilitating *in vivo* detection (33), the creation of a luciferase reporter system in *L. salivarius* strains was targeted. The synthetic Lux operon, containing *luxABCDE*, encoding luciferase (LuxAB) and a fatty acid reductase complex (LuxCDE), was amplified from the pP2lux plasmid (34) and was cloned after the constitutively expressed p44 promoter in the pNZ44 plasmid. Although notable bioluminescence was obtained in the *E. coli* host (Fig. 1), *L. salivarius* cells containing this vector did not generate bioluminescence of sufficient strength to merit its continued use.

DsRed is a popular reporter protein of RFP and is often used as an alternative to GFP due to the generation of a more optimal emission spectrum for fluorescence within complex and live tissues (for a review, see reference 35). The DsRed reporter protein is available commercially as part of a transcriptional fusion with *lacZ* (Clontech). However, when the *dsred* open reading frame (ORF) is moved to another vector, it is often not efficiently expressed and can develop more slowly than GFP (29, 36). To optimize the production of RFP, steps were taken in the oligonucleotide design to reduce the GC content at the start at the 5′ end of the gene, and a typical RBS, ACGAGG, was inserted 8 bp before the translational start site, as suggested previously (29) (see Table S1 in the supplemental material). Despite noticeable fluorescence in *E. coli*, upon transfer of the pNZ44.*rfp* vector into *L. salivarius*, we were, again, unable to detect sufficient fluorescence from fully grown cultures to justify its continued use (Fig. 1).

GFP is a highly useful, stable, and species-independent fluorescent reporter that, unlike bioluminescent reporters, does not require the addition of specific substrates (other than molecular oxygen) for reaction efficiency. Following synthesis, GFP requires an autocatalytic reaction creating a fluorophore by oxidation (37). Previous work has established that GFP can be used as a reporter in *L. salivarius* cells (38). In this study, the *gfp* gene with a corresponding RBS site (AGGAGG) was cloned downstream of the constitutive *Lactococcus lactis* p44 promoter, and fluorescence was observed. The p44 promoter was replaced with a constitutive *Lactobacillus* promoter, p*cysK* (from *L. salivarius* NCIMB 40829 [LSUCC118]), and although the level of fluorescence observed was indeed higher, it was reasonably comparable to that detected with the p44 promoter; therefore, continued use of the lactococcal promoter fusions as a positive control for GFP expression was sufficient (Table 2; Fig. 1).

Notably, although GFP is a very useful reporter *in vitro*, *in vivo* analyses using *L. salivarius* gfp^+ cells were not successful previously due to background tissue (and indeed food) autofluorescence (unpublished data). Therefore, *in vitro* tests were relied on for investigation of the impact of environmental stresses on promoter activity.

***In silico* analysis of bacteriocin promoter regions.** The bacteriocin clusters associated with bactofencin A, salivaricin P, salivaricin T, and ABP-118 were examined in order to identify promoter sequences preceding the genes encoding the bacteriocin structural peptides (Fig. 2). Specifically, regions were examined for typical sigma 70 motifs (−10 [TATAAT] and −35 [TTGACA] sequences) and direct repeats by using manual annotation and promoter-mining software. Directly upstream of the bactofencin A start codon was a predicted sigma 70 promoter with −10 and −35 motifs located 16 bp apart and 23 bp upstream of the likely RBS site (Fig. 2a). A fragment of 110 bp incorporating this intergenic region was amplified and was fused directly to the *gfp* gene in the pNZ44 plasmid to construct *bfnA*prom.*gfp* (Table 2).

Analysis of the regions upstream of the prepeptide and structural genes in the class IIb bacteriocins did not reveal correspondingly obvious promoter regions. Alignment of the intergenic regions from the gene clusters highlights very high sequence similarity (Fig. 2b to d), with 98 to 99% nucleotide identity across the putative promoter regions for these bacteriocins, and therefore, in certain cases, the same constructs were used to represent one putative salivaricin promoter region (described in Table 2). In all, four regions, including an ABP118-specific region of 345 bp upstream of the ABP-118 prepeptide and three promoter regions (*sal*prom1 to *sal*prom3) ranging from 151 bp to 847 bp, representing three regions upstream of the structural genes of salivaricin P and salivaricin T, were amplified (Fig. 2b to d). *sal*prom3 represents a small putative promoter region (see Fig. S1 in the supplemental material) upstream of the bacteriocin prepeptide, whereas the larger regions (*sal*prom1 and *sal*prom2) were included to ensure the cloning of promoter regions that may be located further upstream (Table 2).

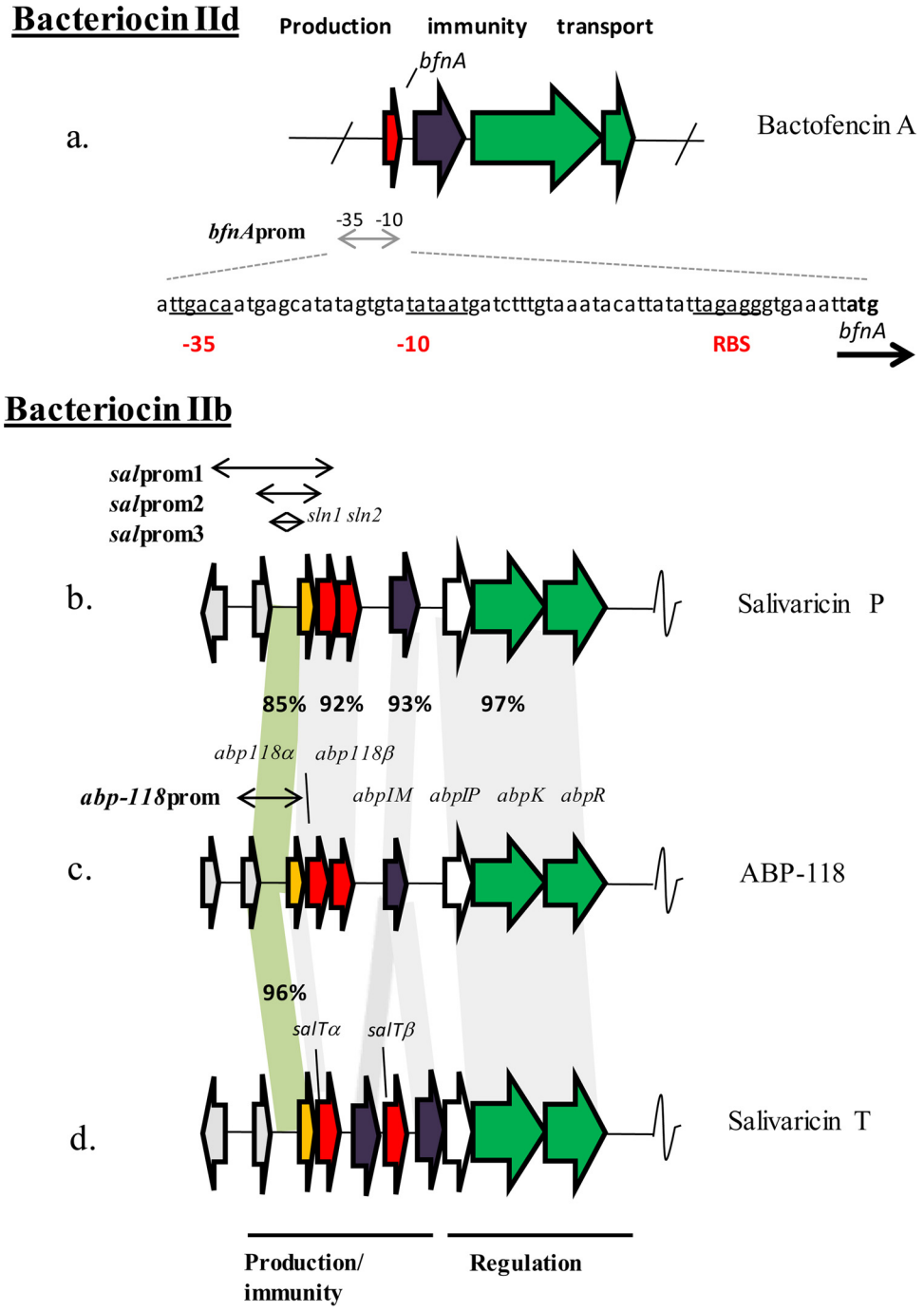

FIG 2 (a) *In silico* analysis of the bactofencin A putative promoter region upstream of the bactofencin structural gene. (b through d) Alignment of the structural genes, immunity genes, and intergenic regions of the class IIb bacteriocins salivaricin P (b), ABP-118 (c), and salivaricin T (d). Red arrows, bacteriocin structural genes; orange arrows, genes encoding bacteriocin prepeptides; green arrows, regulatory genes/transport genes; white arrows, genes encoding bacteriocin-inducing peptides; purple arrows, bacteriocin immunity genes. The green blocks between clusters represent areas within the promoter regions that have high percentages of nucleotide identity. The gray blocks represent regions with high percentages of nucleotide identity in the surrounding genes. The percentages of nucleotide identity are shown.

Care was taken in the cloning of the larger regions to reduce the number of translated nucleotides included in the final constructs. To create a bank of *gfp*$^+$ strains, the promoter-*gfp* fusions were transformed (in most cases; see Table S2 in the supplemental material) into each of the 4 bacteriocin-producing strains: *L. salivarius* DPC6502 (producing bactofencin A), *L. salivarius* DPC6488 (producing salivaricin T and salivaricin L), *L. salivarius* DPC6189 (producing salivaricin P and bactofencin A), and *L. salivarius* NCIMB 40829 (LSUCC118) (producing ABP-118). In addition, a promoterless construct containing the *gfp* gene was also

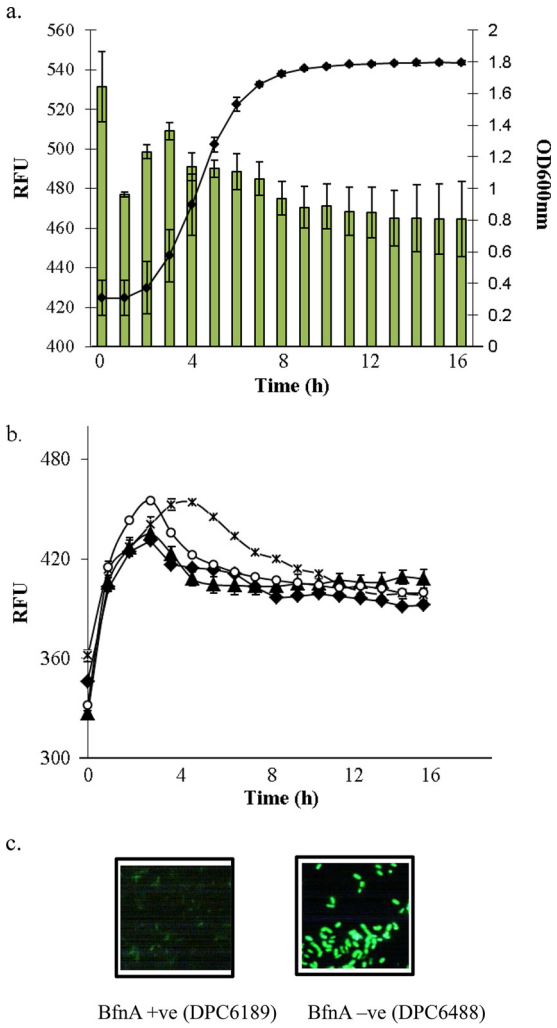

FIG 3 (a) Total fluorescence (measured in RFU) produced by the *bfnA*prom.*gfp* plasmid in the bactofencin A producer *L. salivarius* DPC6502 (green bars) and analysis of the growth of *L. salivarius* DPC6502 over time as measured by the OD at 600 nm. (b) Total fluorescence produced by the *bfnA*prom.*gfp* plasmid in the bacteriocin producers *L. salivarius* LSUCC118 (○), DPC6502 (◆), DPC6189 (▲), and DPC6488 (×). (c) Images of *L. salivarius* DPC6189 (BfnA-positive) and *L. salivarius* DPC6488 (BfnA-negative) cultures expressing *bfnA*prom.GFP under a fluorescence microscope.

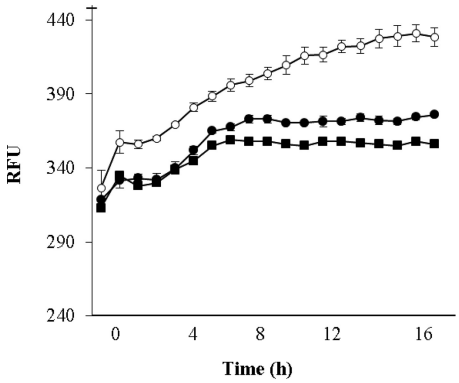

FIG 4 Total fluorescence (measured in RFU) produced by *sal*prom2.*gfp* in *L. salivarius* LSUCC118 in MRS broth and in response to 10^{-5} M ABP-118/SalT IP. ■, *gfp* negative control; ●, *sal*prom2 in MRS broth; ○, *sal*prom2 in MRS broth with IP.

made in each background to serve as a negative control (Table 2; see also Table S2).

***In vitro* analysis of the promoter region of the class IId bacteriocin bactofencin A.** The strengths of the putative promoter regions from the bacteriocin clusters were assessed by using GFP as a reporter. Initially, each bacteriocin promoter was analyzed in the natural background where the corresponding bacteriocin is produced. Fluorescence was compared by visualizing stationary-phase cells under a fluorescence microscope, followed by a comparison of expression monitored over 20 h of growth (see Fig. S2 in the supplemental material). Cloning of the promoter region *bfnA*prom highlighted strong promoter activity initially in the *L. salivarius* DPC6502 (bactofencin A-producing) background, and this activity increased further during the logarithmic growth phase (Fig. 3a). It should be noted here that time zero RFU readings are higher than expected; this is possibly due to an initial adjustment of the instrument.

The analysis revealed also that although the bactofencin A promoter was switched on in all 4 backgrounds tested, GFP was expressed at a lower level in the backgrounds where bactofencin A is naturally produced (i.e., *L. salivarius* DPC6502 and *L. salivarius* DPC6189) than in the backgrounds where the bacteriocin is not encoded (i.e., *L. salivarius* NCIMB 40829 [LSUCC118] and *L. salivarius* DPC6488). This was evident during growth analysis in log phase (Fig. 3b) and also when washed cells were viewed under a fluorescence microscope (Fig. 3c).

***In vitro* analysis of the promoter regions of the structural genes of class IIb bacteriocins.** The predicted promoter regions (*sal*prom1 to *sal*prom3, representing both the salivaricin P and salivaricin T regions, and *abp-118*prom, representing ABP-118) were expressed as GFP fusions in each of the salivaricin-producing backgrounds in order to analyze promoter activity (see Table S2 in the supplemental material). In all instances, these promoters exhibited weak expression during growth analysis in MRS broth (represented by *sal*prom2 in Fig. 4). Indeed, relative fluorescence values (expressed in RFU) resembled those of the negative (promoterless GFP) control (Fig. 4). No increase in promoter activity was observed with either *sal*prom1 or *sal*prom3 in the *L. salivarius* DPC6189 background (not shown). It was thought that this may be due to an insufficient level of IP production, so the predicted inducing peptides for salivaricin P and for salivaricin T/ABP-118 (the genes corresponding to the IPs for salivaricin T and ABP-118 show 100% nucleotide identity) were synthesized and were added at varying concentrations, and fluorescence was monitored over 20 h. The addition of the salivaricin T/ABP-118 IP at 10^{-5} M to cells expressing the *sal*prom2 and *abp-118*prom promoter regions resulted in an increase in fluorescence over time (represented by *sal*prom2 in Fig. 4). The use of the ABP-118 IP to induce bacteriocin activity in *L. salivarius* NCIMB 40829 (LSUCC118) has been reported previously (11).

Bacteriocin promoter activity under simulated environmental conditions. A number of challenge assays were undertaken to establish whether environmental signals, in particular those associated with the GIT, could induce bacteriocin promoter activity, with a view to determining if the bacteriocins might still be produced under the stressful conditions present in the gut or if infor-

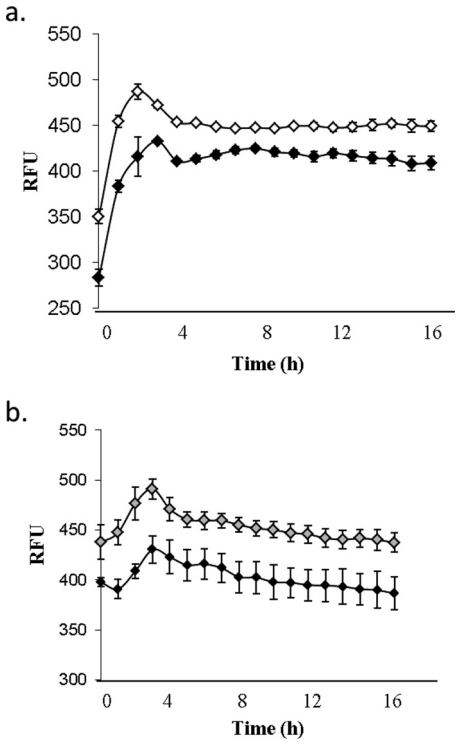

FIG 5 Total fluorescence (measured in RFU) produced by *bfnA*prom.*gfp* in *L. salivarius* DPC6502 in response to salt (a) and to simulated GI fluid (b). Filled symbols, *bfnA*prom.*gfp* in MRS broth; open symbols, *bfnA*prom.*gfp* in MRS broth with 0.5% (wt/vol) NaCl; shaded symbols, *bfnA*prom.*gfp* in MRS broth following a 5-min exposure to simulated GI fluid.

mation could be gained that would facilitate enhanced bacteriocin production in a processing environment. Cells containing the promoter-*gfp* fusions were challenged with low pH, salt, or bile at subinhibitory levels, with the presence of target microbes, or with low levels of simulated gastric juice and were analyzed for promoter activity. For each challenge assay, specific promoter fusions were analyzed in their own bacteriocin-producing backgrounds. The bactofencin A promoter was functional under all conditions tested, including low pH (5.5), the presence of bile (0.2% [wt/vol]), or the presence of target microbes, with GFP expression comparable to that detected in *L. salivarius* strain DPC6502 in lab media (data not shown). In the presence of low levels of added salt (0.25 to 0.5% [wt/vol]), promoter activity actually increased, and GFP levels were higher than those for controls (Fig. 5a). Cells were also exposed for short times (30 s, 1 min, and 5 min) to gastric fluid [pH 2], mimicking the conditions of the upper intestinal tract. Under these stressful conditions, GFP expression increased, indicating induction of *bfnA*prom upon exposure to harsh conditions such as those encountered in the gut (Fig. 5b).

The class IIb bacteriocin promoter regions were also tested in response to the environmental stresses described above; however, no significant increase in promoter activity was observed (data not shown).

DISCUSSION

Bacteriocins have great potential as therapeutic agents that can be used to inhibit important pathogens in food and/or clinical settings. The level at which bacteriocins are produced under stressful environmental conditions has not been extensively explored. In this study, we used a stable expression system for monitoring the activity of *L. salivarius*-associated bacteriocin promoters under various environmental conditions.

This study initially analyzed 3 different potential reporter systems for the study of promoter activity in *L. salivarius* cells. The GFP reporter was found to be more favorable in the bacteriocin-producing backgrounds than either the RFP or the Lux system. Interestingly, a very recent study has reported the development of an optimized GFP variant suitable for anaerobic environments (39); the strains expressing GFP in the current study were analyzed under microaerobic conditions to facilitate fluorescence detection. Both the RFP and Lux systems were also investigated due to their usefulness in live tissues. However, neither system facilitated efficient reporter expression in *L. salivarius*. This is in accordance with previous work on the Lux system, where it was suggested that the *L. salivarius* genome does not contain the genetic machinery to support the light-emitting reaction. The authors suggested that *L. salivarius* lacks a NAD(P)H:FMN oxidoreductase, which functions to synthesize reduced flavin mononucleotide ($FMNH_2$), required for the emission of bioluminescence in the presence of oxygen (38). There are limited studies on the use of the DsRed (RFP) protein in lactic acid bacteria; however, a recent study detailed the successful use of the mCherry red monomer reporter for promoter analysis in *Lactobacillus acidophilus* (40). In this work, the DsRed (RFP) protein was initially optimized for expression in *E. coli* but was not efficiently expressed in *L. salivarius*. This may be due to weak RBS initiation in the *Lactobacillus* background, but further analysis would be required to determine this.

Assessment of fusions of the promoter of the class IId bacteriocin bactofencin A with *gfp* revealed a strong promoter active under all environmental stresses, the activity of which could be increased under specific conditions and upon introduction into non-bactofencin A-producing strains (Fig. 3). It may be that, as for some other bacteriocin clusters, the associated promoter is switched on at a basal level under all conditions, but once the bacteriocin peptide reaches a certain threshold, promoter activity is switched off or repressed (see Fig. S3 in the supplemental material). The genetic determinants for such a bactofencin A-associated regulatory pathway have yet to be identified, but now that the existence of such a mechanism has been indicated, it will be the focus of further studies. In addition, the increased promoter activity upon exposure to mild stresses, such as low levels of salt and simulated gastric fluid (Fig. 5), that may mimic the conditions of the GIT suggests the potential to use this bacteriocin to inhibit gut pathogens *in vivo*. Given that gastric fluid contains salt, we cannot rule out the possibility that salt also plays a key role in promoter induction in this environment.

Analysis of the genomic regions upstream of the structural genes of the 3 class IIb bacteriocins salivaricin P, salivaricin T, and ABP-118 did not reveal obvious promoter regions. However, these promoter regions are likely to be bound by a response regulator from a two-component system and therefore may not contain typical sigma 70 promoter motifs. In addition, it should be noted that in low-GC organisms, stretches resembling −10 elements can be frequent, and therefore, definitive identification of promoter elements can be challenging. The *in vitro* assays investigating the promoters of the class IIb bacteriocins revealed apparent weak expression. Given that addition of the IP stimulated pro-

moter activity (Fig. 4), it is likely that the full promoter region is indeed cloned and active but sensitive to the levels of IP available.

In conclusion, this study investigated the activities of the promoters of bacteriocin production in *L. salivarius*. The results highlight the capacity for salt (bactofencin A) or an inducing peptide (class IIb salivaricins) to induce promoter activity, information that can be used to increase bacteriocin production in the GIT or in the processing environment.

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